Amphiphilic biopolymers (amphibiopols) as new surfactants for membrane protein solubilization

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Abstract

The aim of this study was to develop new surfactants for membrane protein solubilization, from a natural, biodegradable polymer: the polysaccharide pullulan. A set of amphiphilic pullulans (HMCMPs), differing in hydrophobic modification ratio, charge ratio, and the nature of the hydrophobic chains introduced, were synthesized and tested in solubilization experiments with outer membranes of *Pseudomonas fluorescens*. The membrane proteins were precipitated, and then resolubilized with various HMCMPs. The decyl alkyl chain (C_{10}) was the hydrophobic graft that gave the highest level of solubilization. Decyl alkyl chain-bearing HMCMPs were also able to extract integral membrane proteins from their lipid environment. The best results were obtained with an amphiphilic pullulan bearing 18% decyl groups ($18C_{10}$). Circular dichroism spectroscopy and membrane reconstitution experiments were used to test the structural and functional integrity of $18C_{10}$-solubilized proteins (OmpF from *Escherichia coli* and bacteriorhodopsin from *Halobacterium halobium*). Whatever their structure type (α or β), $18C_{10}$ did not alter either the structure or the function of the proteins analyzed. Thus, HMCMPs appear to constitute a promising new class of polymeric surfactants for membrane protein studies.

Keywords: Membrane protein; solubilization; amphiphilic polysaccharide; polymeric surfactant; pullulan

Integral membrane proteins play key roles in various biological processes (Tsukihara and Lee 1999) including photosynthesis, respiration, and bacterial resistance to antibiotics. Their hydrophobicity and insolubility in water render them much more difficult to study than soluble proteins, and detergents are required to facilitate their handling in aqueous solutions. However, the use of detergents in biochemical and biophysical experiments may cause various problems. For example, detergent micelles are in thermodynamic equilibrium with monomers that may affect protein structure and/or function (Breyton et al. 1997). They may also perturb analytical techniques (Seigneuret et al. 1991; Le Maire et al. 2000). The choice of a detergent is not always evident, and depends on the system considered (Hjelmeland and Chrambach 1984). A large variety of detergents is therefore required to deal with the various situations that may arise.

Current research on monomeric detergents focuses on the improvement of specific features: the design of less flexible amphiphilic molecules to favor crystallization (Yu et al. 2000); fluorinated detergents that bind less efficiently to proteins, limiting dissociation and inactivation (Chabaud et al. 1998; Barthélémy et al. 1999); synthetic sugar esters that can be removed by hydrolytic cleavage (Peters et al. 2000); and lower cost analogs of efficient but expensive detergents (Hildreth 1982; Plusquellec et al. 1989).

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Abbreviations: BR, bacteriorhodopsin; HMCMP, hydrophobically modified carboxymethylpullulan; LiDS, lithium dodecylsulfate; Octyl-POE, octylpolyoxyethylene; Tris-HCl, Tris(hydroxymethyl)aminomethane hydrochloride.

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Another approach involves reducing the thermodynamic instability of micelles by constructing a similar assembly with surfactant chains covalently linked together (Liu et al. 1999), thereby limiting exchange in micelles (Borisov and Halperin 1998) and excluding small molecules able to destabilize protein structure. This can be achieved with amphiphilic polymers, sometimes called "polymeric surfactants." The first attempts to use polymers to keep membrane proteins in a soluble state involved a synthetic amphiphilic peptide ("peptitergent"). This compound maintained bacteriorhodopsin and rhodopsin in aqueous solution (Schafmeister et al. 1993) but caused a loss of activity of the Na^+/K^+ exchanging ATPase (Soomets et al. 1997).

A class of amphiphilic polymers called amphipols (Tribet et al. 1996) was successfully developed for handling membrane proteins in aqueous solutions. Amphipols are low molecular weight polyacrylates, hydrophobically modified with octyl groups. They have been shown to stabilize several integral membrane proteins in their native states in aqueous solution. Amphipol/protein complexes appear to be irreversible over the time scale of the experiments (Tribet et al. 1997) and can be successfully analyzed by scanning transmission electron microscopy (Tribet et al. 1998). However, a more detailed study of the effects of amphipols on membrane protein function (Champeil et al. 2000) showed that the activity of Ca^{2+}-ATPase is low in the presence of amphipol alone but increases in the presence of detergent. This polymer is also unable to solubilize membranes, so the proteins must first be solubilized with detergents, and then the detergent replaced with amphipol. Recent developments have nevertheless demonstrated the potential of a zwitterionic amphipol with dodecyl side chains to support full activity of diacylglycerol kinase in the absence of additional lipid or detergent (Nagy et al. 2001; Gorzelle et al. 2002).

In recent years, we have described a number of chemical modifications to polysaccharides, in particular pullulan (Bataille et al. 1997; Glinel et al. 1999). As detergents derived from glucose and maltose are effective (Engel et al. 2002), one way of improving amphipols could be to develop new amphiphilic pullulan derivatives, able to solubilize membrane proteins from lipid bilayers, while preserving their activity. These macromolecules would also have the advantage of having a natural and biodegradable hydrophilic backbone, and could thus be named "amphibiopols."

A set of hydrophobically modified carboxymethylpullulans (HMCMPs, Fig. 1) of medium molar mass ($\overline{M_n} \approx 30{,}000\ g \cdot mole^{-1}$) and narrow size distribution were synthesized (Duval et al. 2001; Duval-Terrié et al. 2002). They differed in the nature of the hydrophobic chain, in hydrophobic modification ratio (number of hydrophobic groups per 100 anhydroglucose units), and in charge ratio (number of ungrafted carboxymethyl groups per 100 anhydroglucose units). We identified two different critical concentrations, demonstrating that HMCMPs display more complex behavior than classical molecular surfactants in dilute aqueous solution.

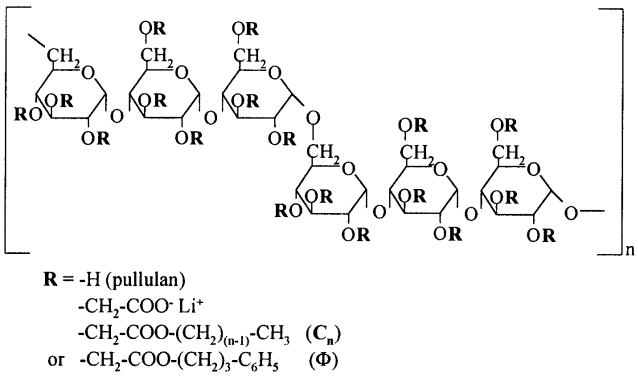

Figure 1. Formulas of hydrophobically modified carboxymethylpullulans (HMCMPs).

In this study, we investigated the solubilization capacities of this set of amphiphilic pullulan derivatives. We tested the various HMCMPs on bacterial outer membranes, and identified the most appropriate HMCMP for the desired solubilization. Experiments were then performed to evaluate the effect of this amphiphilic polymer on protein structure and function.

Results

Development and improvement of HMCMPs for membrane protein solubilization

The main part of this study was devoted to the development of hydrophobically modified pullulans (HMCMPs) able to solubilize integral membrane proteins. Comparison of a set of HMCMPs differing in hydrophobic modification ratio, charge ratio, and type of hydrophobic chain (Table 1), was

Table 1. *Characteristics of HMCMPs*

Name	Hydrophobic modification ratio[a] (%)	Charge ratio[b] (%)
CMP	0	92
$18C_8$	18	5
$18C_{10}$	18	91
$23C_{10}$	23	86
$31C_{10}$	31	78
$43C_{10}$	43	77
$16C_{12}$	16	76
10Φ	10	110

[a] Number of hydrophobic chains per 100 anhydroglucose units.
[b] Number of ungrafted carboxymethyl groups per 100 anhydroglucose units.
C_8, C_{10}, and C_{12}, respectively refer to octyl, decyl, and dodecyl linear alkyl groups.
Φ = 3-phenylpropyl group.

necessary to identify the most appropriate parameters. HMCMPs are denoted by $\boldsymbol{x}C_n$, where n is the number of carbon atoms in the grafted alkyl chain, or by $\boldsymbol{x}\Phi$ if the hydrophobic chain added consisted of 3-phenylpropyl groups. x is the hydrophobic modification ratio (number of hydrophobic chains per 100 anhydroglucose units).

The comparison of HMCMPs was based on their ability to solubilize proteins from the outer membranes of the bacterium *Pseudomonas fluorescens.*

We first investigated the influence of the nature and length of the hydrophobic chain introduced by comparing HMCMPs with similar hydrophobic modification ratios but different kinds of hydrophobic chain. Modification ratios were not exactly identical due to chemical modification constraints. The HMCMPs were tested on precipitated proteins from outer membranes. To compare the solubilization capacities of the different HMCMPs, the total amount of protein in the supernatant after ultracentrifugation was determined by UV spectroscopy. Two references were used in each experiment: detergent-free Tris-HCl buffer, to check for the absence of water-soluble protein and set the absorbance corresponding to 0% hydrophobic membrane protein solubilization, and a 5 $g \cdot L^{-1}$ LiDS solution, taken as the reference for 100% solubilization. This detergent, although denaturing, is highly efficient at solubilizing membrane proteins: All the proteins initially present in the membranes were solubilized in 5 $g \cdot L^{-1}$ LiDS.

The amount of protein remaining in the supernatant after ultracentrifugation is shown in Figure 2. All four HMCMPs studied maintained some of the hydrophobic proteins in aqueous solution. The comparison shown is that for a concentration of 2 $g \cdot L^{-1}$, but a similar trend was observed with a concentration of 5 $g \cdot L^{-1}$: $18C_8$ displayed a very low solubilization capacity, 10Φ was more efficient and the best results were obtained with $18C_{10}$ and $16C_{12}$. We found that $18C_{10}$ was slightly more efficient than $16C_{12}$, which is less

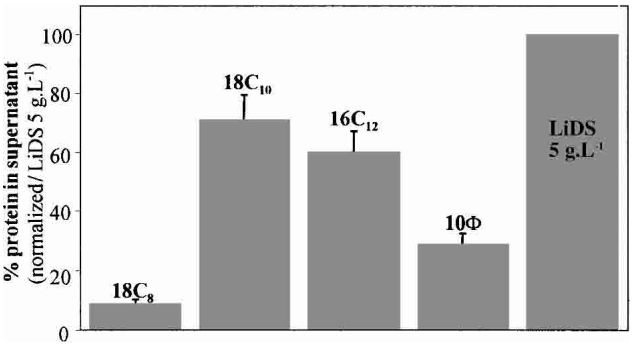

Figure 2. Influence of the hydrophobic chain of HMCMPs on solubilization efficiency. Amount of protein in the supernatant determined by measuring UV absorption at 280 nm for various HMCMPs (used at a concentration of 2 $g \cdot L^{-1}$) and 5 $g \cdot L^{-1}$ LiDS. Values are means for three sets of experiments with precipitated outer membranes from *Pseudomonas fluorescens.*

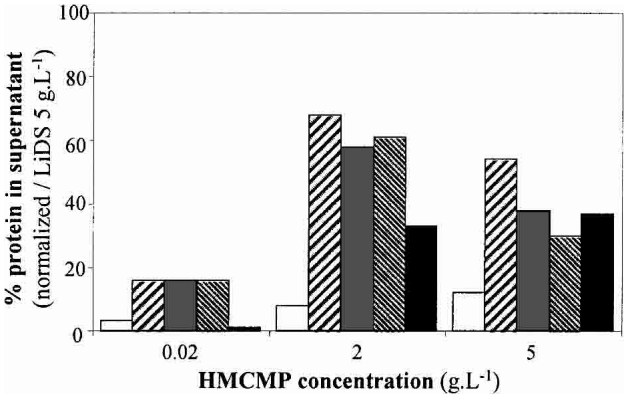

Figure 3. Influence of hydrophobic modification ratio of HMCMPs. Amount of protein (from outer membranes of *Pseudomonas fluorescens*) in the supernatant determined from UV absorbance at 280 nm for xC_{10}. Open column: CMP; wide slanted bar column: $18C_{10}$; densely dotted column: $23C_{10}$, narrow slanted bar column: $31C_{10}$; filled column: $43C_{10}$.

soluble. Indeed, a few hours of stirring were required to obtain a homogeneous solution of $16C_{12}$, and at high concentration some of this amphiphilic polysaccharide precipitated upon ultracentrifugation, which was not the case for $18C_{10}$. Thus, HMCMP bearing decyl groups seemed to be the most useful of the polymers tested for solubilizing membrane proteins. Subsequent experiments were performed with HMCMPs bearing decyl chains.

Amphiphilic pullulans bearing decyl groups (xC_{10}) were tested in realistic conditions of detergent use, without prior precipitation of membranes. xC_{10} with various proportions of decyl groups (up to 43%) were compared at various concentrations. Higher hydrophobic modification ratios resulted in insoluble amphiphilic polymers.

Ungrafted CMP was inefficient at protein solubilization, as expected (Fig. 3). In contrast to the results obtained with noncarboxymethylated pullulan (not shown), non-negligible amounts of protein were nevertheless detected, implicating electrostatic interactions in the solubilization mechanism. The poor efficiency of $43C_{10}$ almost certainly results from its low solubility. Decyl-bearing HMCMPs with intermediate modification ratios presented similar profiles of behavior as a function of concentration: The amount of protein extracted initially increased with polymer concentration, reached a maximum, and then decreased at high polymer concentration. This decrease, which was associated with the visible sedimentation of the polymer upon ultracentrifugation, occurred earlier for polymers with higher hydrophobic modification ratios.

In this experiment it was clear that the most appropriate xC_{10} for the solubilization of membrane proteins from *P. fluorescens* was $18C_{10}$, used at a concentration of 2 $g \cdot L^{-1}$; in that case, 68% of proteins were solubilized.

Optimization of HMCMPs was based on their ability to solubilize integral membrane proteins from outer mem-

branes of *P. fluorescens*. To investigate the potential of this new polymeric surfactant more generally, $18C_{10}$ was also tested on outer membranes from *E. coli* (not shown): 57% of membrane proteins were solubilized, indicating that $18C_{10}$ is able to extract integral membrane proteins efficiently from different species.

Structure and function of $18C_{10}$-solubilized proteins

The protein samples used in the experiments described above contained a mixture of proteins and could not easily be used to evaluate the effect of $18C_{10}$ on the structure and function of the proteins solubilized. It was therefore necessary to work with single, purified proteins, obtainable in a detergent solution. Two kinds of integral membrane protein were used to study the effect of $18C_{10}$ on protein structure and function: OmpF porin, with a major β-sheet structure, and an α-helical protein, bacteriorhodopsin (BR).

As for peptitergents (Schafmeister et al. 1993) and amphipols (Tribet et al. 1996), we used a "dilution" protocol: A solution of purified protein in detergent was diluted in either detergent-free buffer, or $18C_{10}$ or detergent solutions. The dilution factor is such that dilution in detergent-free buffer resulted in precipitation of almost all the protein present whereas, in the presence of 2 $g \cdot L^{-1}$ $18C_{10}$, 100% of initial OmpF and 40% of BR remained soluble.

The structure and function of $18C_{10}$-solubilized proteins were compared with those of proteins in their original detergent, which has been shown to preserve these aspects (Garavito and Rosenbusch 1986; Meyer et al. 1992; Saint et al. 1996).

Circular dichroism experiments were carried out to investigate the influence of $18C_{10}$ on the structural properties of OmpF and BR (Fig. 4). Preliminary experiments showed that the use of HMCMP was compatible with these spectropolarimetric assays. The only observed drawback in this study was the difficulty in obtaining accurate measurements in the far UV range for BR solutions. Indeed, BR was originally solubilized in Triton, and it was almost impossible to record interpretable spectra below 200 nm with this detergent. We therefore used data corresponding to 200 to 240 nm for secondary structure determination. The spectra obtained with the two solubilizing agents ($18C_{10}$ and reference detergent, either Octyl-POE for OmpF or Triton for BR) were almost identical (Fig. 4). For OmpF, the spectrum with a negative band between 215–220 nm (Fig. 4A) is typical of β-sheet spectra encountered for bacterial porins with an estimated β-sheet content around 60%. For BR (Fig. 4B), the spectrum displayed two negative CD bands at 208 and 222 nm, characteristic of a highly helical structure with an estimated helical content of 65%.

The channel-forming activity of OmpF was checked by reconstitution in planar lipid bilayers. Before these experiments, the $18C_{10}$ polymer was added to measurement cells in the same conditions as protein samples (concentration ≤ 2 $mg \cdot L^{-1}$). It induced neither lipid bilayer rupture nor channel formation. Membrane leakage was detected only at final polymer concentrations 40 times higher than those used in the experiment.

We then investigated the functional characteristics of the $18C_{10}$-solubilized OmpF. On reconstitution in an asolectin bilayer bathed with 1 M NaCl (pH 7.4), this solubilized

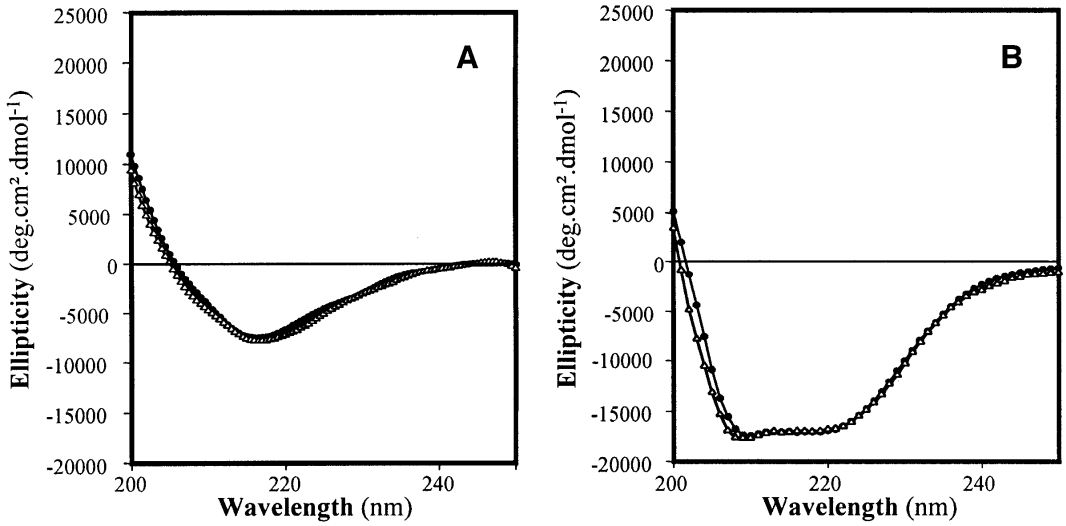

Figure 4. Circular dichroism spectra of OmpF and bacteriorhodopsin. (*A*) CD spectra of OmpF in 5 $g \cdot L^{-1}$ Octyl-POE (filled circles) or in 2 $g \cdot L^{-1}$ $18C_{10}$ (open triangle) solutions. (*B*) CD spectra of BR in 5 $g \cdot L^{-1}$ Triton X-100 (filled circles) or in 2 $g \cdot L^{-1}$ $18C_{10}$ (open triangles). Each spectrum was acquired with 0.02-cm path length cells, between 200 and 250 nm, in steps of 0.5 nm and with an integration time of 2 sec.

porin displayed a reconstitution efficiency similar to that of Octyl-POE-solubilized OmpF, and typical single-channel activity (Schindler and Rosenbusch 1978; Saint et al. 1996): opening of the trimeric form with a conductance value of (2400 ± 60) pS and closure as successive monomers with a conductance value of (810 ± 30) pS (Fig. 5A) if the applied potential was high enough. I-V curves and voltage ramps of ±200 mV were used to determine the critical voltage (V_c), characteristic of the voltage-gated behavior of the OmpF porin (Schindler and Rosenbusch 1978; Lakey and Pattus 1989; Saint et al. 1996). OmpF porin was added to the measurement cell and the voltage-dependent closing of the channels was measured (Fig. 5B): $V_c = -(130 \pm 10)$ mV in the negative quadrant and $V_c = +(140 \pm 10)$ mV in the positive quadrant.

The denaturation of BR was checked by recording its absorption spectrum between 350 and 700 nm. The native purple membrane of *H. halobium* displays maximal absorption at 558 nm, whereas the peak in absorption occurs at 390 nm if BR is denatured (London and Khorana 1982; Plusquellec et al. 1989). When BR was solubilized with $18C_{10}$, the absorption spectrum was similar to that of the native BR, with a slightly shifted absorption maximum (550 nm). This corresponds to a "detergent-induced" blue shift, which is currently observed with mild detergents such as Hecameg (Plusquellec et al. 1989) or Triton X-100 (London and Khorana 1982), and also with amphipols (Tribet et al. 1996). Moreover, no absorption was recorded at 390 nm. These results show that the native form of BR is preserved when solubilized with $18C_{10}$.

Discussion

Our primary aim was to develop amphiphilic pullulans able to solubilize membrane proteins. Our strategy involved the synthesis of a set of amphiphilic pullulan derivatives and comparison of their performances to identify the polymer that solubilized membrane proteins most efficiently.

The hydrophobic modification of pullulan consisted of the addition of alkyl groups but, in contrast to amphipols, these groups were grafted onto the polysaccharide by means of an ester bond, which is easy to hydrolyze if protein release is required. Furthermore, in contrast to what has been observed for amide bonds, ester bonds do not interfere with protein assays based on a reaction with peptide linkages. We also introduced carboxymethyl groups to ensure the solubility in water of highly hydrophobically modified polymers.

Membrane protein solubilization with classical detergents (Jones et al. 1987) may be viewed as involving two major steps (for details, see Le Maire et al. 2000): insertion of detergent into the lipid bilayer, and then solubilization of the protein, thanks to the formation of a detergent monolayer, with an intermediate phase in which mixed lipid–protein–detergent complexes are formed. To improve HMCMPs, we initially focused on the final phase, using precipitated outer membranes. Precipitation leads to the removal of some of the lipids, thereby facilitating the removal of proteins from lipid bilayers.

Based on the amphipol model (Tribet et al. 1996), we first synthesized carboxymethylpullulans bearing octyl groups, but these compounds were inefficient at solubilization. We therefore investigated more hydrophobic grafted chains: longer alkyl groups (decyl and dodecyl) and phenylpropyl groups likely to establish specific interactions with hydrophobic sites on the protein. We found that the nature of the hydrophobic chain had a major effect on the solubilization efficiency of HMCMPs (Fig. 2). The ability of HMCMPs with alkyl chains to maintain highly hydrophobic proteins in aqueous solution increases with increasing chain length

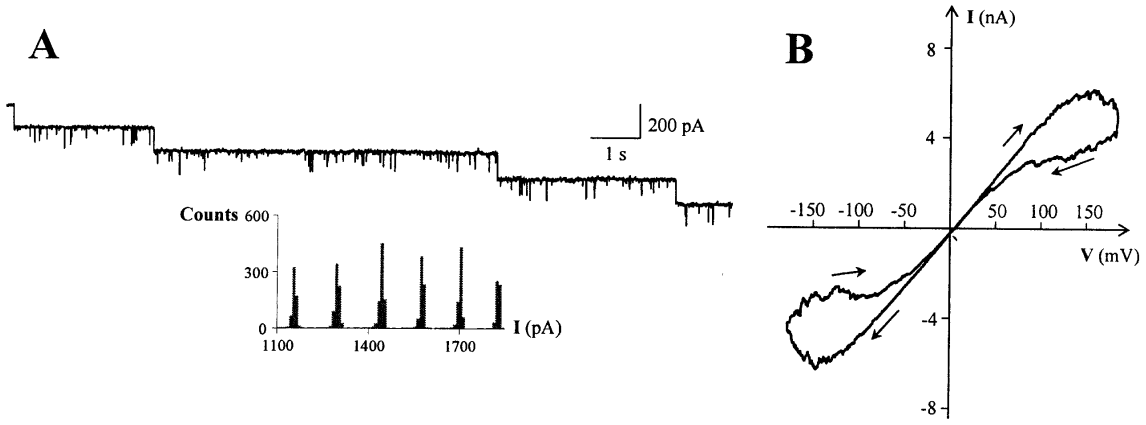

Figure 5. Channel properties of the *E. coli* OmpF solubilized by the $18C_{10}$ polymer after incorporation into planar lipid bilayers. (*A*) Single-channel activity, that is, the closure of OmpF as successive monomers, in buffered 1 M NaCl, with an applied potential of –170 mV. The associated histogram measures a conductance level of 810 pS. (*B*) Current–voltage curve obtained by applying a ramp potential across the bilayer from 0 to ±200 mV, at 2 mV · sec^{-1}, with the same subphase.

(from C_8 to C_{10}), and therefore, with increasing hydrophobicity. Nevertheless, increasing alkyl chain length also decreases the solubility of the amphiphilic pullulan derivative, resulting in a decrease in solubilization efficiency (beyond C_{10} chains). Hence, a compromise between solubilization efficiency and polymer solubility is required, and in our experiments, decyl groups appeared to be the most suitable. This is consistent with previous data on sulfoxide surfactants showing that the extraction of proteins from membranes depends on the length of the hydrophobic chain and seems to reach a maximum with a decyl hydrocarbon chain (Barthélémy et al. 1998). However, 10 carbons is not the optimum alkyl chain length in all cases: The hydrophobic group giving optimal solubilization depends on the hydrophilic part of the polymer, whereas both parts of the amphiphilic macromolecule influence the hydrophilic/hydrophobic balance. Indeed, in the case of copolymers of maleic acid anhydride and vinyl alkyl, copolymers bearing hexyl groups display the strongest binding affinity for a soluble protein, β-lactoglobulin (Gao and Dubin 1998). In other respects, polyoxyethylene glycol or carbohydrate detergents with dodecyl chains are optimal for the solubilization of Ca^{2+}-ATPase in a functional state (Lund et al. 1989). Optimum alkyl chain length may also depend on the protein.

The introduction of phenylpropyl groups was an attractive option, as this kind of hydrophobic chain may establish specific interactions with aromatic residues on the protein surface. However, such HMCMPs did not have particularly useful properties. Despite the presence of aromatic groups, 3-phenylpropyl chains establish very few hydrophobic interactions, probably due to their bulkiness, and the fact that they are shorter and less flexible than linear alkyl chains.

By optimizing alkyl chain length, we were able to synthesize, for the first time, a polymeric agent that can be used to extract proteins from their lipid environment (Fig. 3). To our knowledge, this has not been achieved with amphipols. We attempted to improve the solubilization capacity of HMCMPs bearing decyl chains, by increasing the hydrophobic modification ratio, and so the hydrophobicity of these macromolecules. This strategy was based on a study showing that the binding of bovine serum albumin and hydrophobically modified polyacrylates increases with the extent of hydrophobic modification for a given alkyl dangling group (Porcar et al. 1999).

The low efficiency of unmodified CMP (Fig. 3), demonstrates that hydrophobic modification is essential if the protein is to be kept in a soluble state. However, protein solubilization did not increase as the modification ratio increased in the 18–31% range (for polymer concentrations up to 2 $g \cdot L^{-1}$). Further increases in hydrophobic modification ratio (see $43C_{10}$) even led to a decrease in protein solubilization. This was particularly true if a high modification ratio was combined with a high HMCMP concentration. Increasing the hydrophobic modification ratio has two effects. It increases HMCMP hydrophobicity, which favors hydrophobic interactions with proteins, but also decreases HMCMP solubility (similarly to an increase in hydrophobic alkyl chain length). Moreover, it decreases the charge ratio, as hydrophobic chains are grafted onto carboxymethyl groups. This also results in a decrease in polymer solubility, and reduces the opportunities for electrostatic interactions, which are thought to play an important role in the solubilization mechanism. For these reasons, there is likely to be an optimum degree of modification and, for decyl group-bearing HMCMPs, this degree of modification is 18%, with higher degrees of modification decreasing the amount of protein solubilized.

After identifying $18C_{10}$ as the best HMCMP for solubilizing membrane proteins, we determined the concentration at which it should be used. Increases in solubilization up to a maximum with increasing detergent concentration have been reported for monomeric detergents such as HECAMEG (Plusquellec et al. 1989), tripod amphiphiles (Yu et al. 2000), and for amphipols (Tribet et al. 1996, 1997). The solubilizing capacity of $18C_{10}$ increases with concentration until 2 $g \cdot L^{-1}$, at which a maximum is reached. The decrease in membrane protein solubilization at high HMCMP concentration is probably due to the formation of large aggregates. Thus, the use of $18C_{10}$ at a concentration of 2 $g \cdot L^{-1}$ appears to be optimal for the solubilization of membrane proteins by HMCMPs. However, this result was established for membrane proteins from *P. fluorescens*, and optimum conditions may differ for other membrane proteins. Nevertheless, $18C_{10}$ also efficiently solubilized outer membrane proteins from another bacterium, *E. coli*, although another HMCMP might be more efficient in this case.

After optimizing the HMCMP for solubilization, we evaluated its effects on membrane protein structure and function. Two kinds of integral membrane protein were tested: one with a major α-helical structure (BR) and one with a major β-sheet structure (OmpF). Like amphipols, $18C_{10}$ keeps these different integral membrane proteins soluble in aqueous solution (Tribet et al. 1996).

Neither OmpF nor BR underwent structural modifications in $18C_{10}$, as assessed by secondary structure determination assays using a reference nondenaturing detergent (London and Khorana 1982; Eisele and Rosenbusch 1990). Moreover, the calculated conformations were highly consistent with the reported 3D structures of OmpF and BR (Cowan et al. 1995; Pebay-Peyroula et al. 1997). These two proteins belong to different structural families: all-β for OmpF and major-α for BR. They also possess different physicochemical properties: moderate hydrophobicity for OmpF because only alternate hydrophobic residues are present in the β-sheets embedded in the outer membrane, whereas BR, composed of numerous interacting transmembrane helical segments, is very hydrophobic. Thus the pres-

ervation of the conformation of these two proteins indicates that the HMCMP tested was fully able to maintain the structural integrity of different classes of membrane proteins.

OmpF porin solubilized in $18C_{10}$ was successfully reconstituted into planar lipid bilayers. The functional characteristics of OmpF, including monomeric conductance value (810 pS) and critical threshold potential ($V_c = \pm 130–140$ mV), were not significantly different from those for octyl-POE-solubilized porin (820 pS and $V_c = +155$ mV; Saint et al. 1996). Likewise, the structure of $18C_{10}$-solubilized BR was not affected: The spectral properties of BR were indeed typical of its native form, as was also observed for BR solubilized in solutions of amphipols (Tribet et al. 1996) or Hecameg (Plusquellec et al. 1989).

Some detergents induce changes in conductance or denaturation of the membrane proteins (London and Khorana 1982; Lakey et al. 1985; Champeil et al. 2000), and conservation of the structural and functional characteristics of the integral membrane proteins tested makes the HMCMPs we developed very promising tools.

Finally, we developed hydrophobically modified carboxymethylpullulan derivatives that solubilized membrane proteins from lipid membranes without affecting their structure or function. The degradability of HMCMPs is also a potentially important advantage for membrane biochemistry. If binding between the HMCMP and protein is irreversible, as with amphipols (Tribet et al. 1996), then HMCMP could be degraded (chemically or enzymatically) to facilitate exchange between HMCMP and, for example, lipids (for reconstitution experiments), or another detergent.

Conclusion

In this study we successfully developed new polymeric surfactants for membrane protein solubilization. Unlike other polymers, these hydrophobically modified pullulans (HMCMPs) extracted membrane proteins from their lipid environment and were powerful solubilization agents: $18C_{10}$ extracted 68% of the amount of protein extracted by lithium dodecylsulfate from the outer membranes of *P. fluorescens*. The structure and function of solubilized purified membrane proteins, OmpF and BR, were preserved. HMCMPs also have the advantage of being derived from a natural, biodegradable and biocompatible polysaccharide, thus deserving the appellation "amphibiopols."

These HMCMPs were designed to extract membrane proteins from bacterial outer membranes. However, as the chemical modification of pullulan is well controlled, the composition of HMCMPs is easy to modulate (by varying the kind of hydrophobic chain, hydrophobic modification ratio, ionic charges, etc.). It should therefore be possible to develop different HMCMPs suitable for each particular protein or application. In particular, we are attempting to develop zwitterionic amphibiopols to overcome the possible problems associated with the high anionic net charge of HMCMPs.

Materials and methods

Solutions were buffered at pH 7.4 with 10 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl).

Synthesis and characterization of amphiphilic pullulan derivatives

The hydrophobically modified carboxymethylpullulans (HMCMPs) were synthesized by chemically modifying pullulan (Hayashibara Biochemical Laboratory, Okayama, Japan). Carboxymethyl groups were introduced to generate carboxymethylpullulan (CMP); then some of the carboxylic functional groups were coupled to hydrophobic chains (C. Duval-Terrié, J. Huguet, and G. Muller, in prep.).

The HMCMPs obtained (Fig. 1) had an $\overline{M_n} \approx 30{,}000$ g · mole^{-1} and a rather narrow size distribution (e.g., polydispersity index is 1.1 for $18C_{10}$), as determined from flow-field flow fractionation with on-line coupling multi-angle laser light scattering measurements (Duval et al. 2001). x is the hydrophobic modification ratio, defined as the number of hydrophobic groups per 100 anhydroglucose units.

Bacterial strains, growth conditions, and isolation of bacterial membranes

P. fluorescens MF0 was cultured overnight with shaking, at 28°C in nutrient broth. The cells were then harvested by centrifugation at 8000 × *g* for 10 min at 4°C. Outer membranes were extracted by the spheroplast procedure (Mizuno and Kageyama 1978) with the modifications described by Dé et al. 1995.

Precipitation of proteins from bacterial outer membranes

We precipitated 0.18 mg of protein from *P. fluorescens* outer membranes by incubation with 1.5 mL acetone for 2 h at −18°C. The samples were centrifuged (5 min, 4°C, 17,500 × *g*) and the dried precipitated material was incubated for 1 h at 4°C then washed with 0.5 mL of 1 M trichloroacetic acid (TCA). The insoluble material was washed again with 0.5 mL TCA, then three times with 1 mL water and finally with 1 mL Tris-HCl buffer.

Membrane protein solubilization

Solubilization of proteins precipitated from P. fluorescens *outer membranes*

Precipitated membranes were suspended in 1 mL Tris-HCl and stirred overnight at 4°C to remove all soluble molecules. The samples were centrifuged, the supernatant was removed, and the pellet was suspended in 200 μL of HMCMP solution, Tris-HCl, or one of two reference detergents: lithium dodecyl sulfate (LiDS) and octylpolyoxyethylene (Octyl-POE, Bachem). Samples were incubated overnight at 4°C with magnetic stirring and centrifuged (1 h, 4°C, 180,000 × *g*) to remove insoluble proteins. Supernatants were collected for analysis.

Solubilization of proteins from outer membranes of P. fluorescens

We made up a solution containing 0.20 mg of total protein from *P. fluorescens* outer membranes in 500 μL water. We then centrifuged this solution at 17,500 × *g* for 1 h at 4°C. The pellet was suspended in 500 μL Tris-HCl. The resulting suspension was incubated overnight at 4°C with magnetic stirring to remove all soluble compounds, and then centrifuged as described above. The pellet was then suspended in 200 μL of one of the HMCMP solutions, Tris-HCl buffer, Octyl-POE, or LiDS. The mixture was incubated overnight at 4°C with stirring, and the samples were then centrifuged at 180,000 × *g* for 1 h, at 4°C, and supernatants collected for further analysis.

Solubilization of bacteriorhodopsin from H. halobium

A suspension of purple membrane from *H. halobium* (Oesterhelt and Stoecknenius 1974) (generously provided by D. Lévy, Institut Curie) was added (at 1 $mg \cdot mL^{-1}$ BR) to a 5 $g \cdot L^{-1}$ Triton X-100 solution and incubated overnight at room temperature. Samples were centrifuged at 180,000 × *g* as above, to remove unsolubilized material.

Analysis of solubilized proteins

Total protein content was estimated by UV absorption measurements at 280 nm. Final results are expressed as a fraction of total protein content in the 5 $g \cdot L^{-1}$ LiDS supernatant, taken as the reference. BR was titrated by measuring absorption at 560 nm, assuming a molar extinction coefficient of 63,000 $L \cdot mole^{-1} \cdot cm^{-1}$ (Hwang and Stoekenius 1977).

Functional and structural studies

Purified OmpF from *Escherichia coli* (~1.9 ± 0.2 $g \cdot L^{-1}$ in 3 $g \cdot L^{-1}$ Octyl-POE, gift from N. Saint, Centre de Biochimie Structurale) and BR (~0.53 ± 0.05 $g \cdot L^{-1}$ in 5 $g \cdot L^{-1}$ Triton X-100) were diluted 20-fold with detergent-free buffer, Octyl-POE or Triton X-100, and HMCMP solutions. Samples were incubated overnight at 4°C with magnetic stirring and centrifuged at 180,000 × *g* as described above. Supernatants were collected for analysis. Denaturation of BR was checked by recording its absorption spectrum between 350 and 700 nm.

Circular dichroism spectroscopy

CD spectra were recorded for solutions of OmpF (130 $\mu g \cdot mL^{-1}$ in 5 $g \cdot L^{-1}$ Octyl-POE and in 2 $g \cdot L^{-1}$ $18C_{10}$) and BR (30 $\mu g \cdot mL^{-1}$ in 5 $g \cdot L^{-1}$ Triton X-100 and 11 $\mu g \cdot mL^{-1}$ in 2 $g \cdot L^{-1}$ $18C_{10}$), using a CD6 spectropolarimeter (Jobin-Yvon) routinely calibrated with a standard solution of d-10 camphorsulfonic acid. Experimental data (corresponding to a mean of five scans and corrected for background), expressed as mean molar residue ellipticity, were used for secondary structure determination according to the SELCON method (Sreerama and Woody 1993).

Reconstitution into planar lipid bilayers

Single-channel measurements and voltage-gating experiments were performed as described by Dé et al. (2000). Asolectin IV-S from soybean was used as lipid. The electrolyte solution was 1 M NaCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) pH 7.4. The protein concentration in measurement cells was ~10^{-9} M.

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